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(54) Title: SINGLE CHAIN T-CELL RECEPTOR

(57) Abstract

Disclosed is a single chain T-cell receptor which binds specifically to an MHC peptide ligand. The single chain T-cell receptor is a 3-domain construct comprising an α chain variable domain, a β chain variable domain and a constant domain. Also disclosed is a self-signaling single chain T-cell receptor which binds specifically to an MHC/peptide ligand and mediates signal transduction.

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SINGLE CHAIN T-CELL RECEPTOR

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Background of the Invention

T-cell receptors (TCRs) are recognition molecules present on the surface of T lymphocytes. Although closely related to antibody molecules, T-cell receptors have numerous properties which distinguish them from antibodies. One fundamental difference between T-cell receptors and antibody molecules is that antibody molecules exist in two alternative forms: cell surface molecules or soluble secreted molecules. Native T-cell receptors exists, however, only as cell surface molecules.

The T-cell receptors which are found most commonly on the surface of T-cells are comprised of two glycoprotein subunits which are referred to as the α and β chains. Both chains have a molecular weight of about 40 kDa and possess a variable and a constant domain. The genes which encode the α and β chains are organized in a manner which is similar to antibody gene organization in that there are libraries of V, D and J regions from which the genes are formed by genetic rearrangement.

TCRs recognize antigen which is presented by an antigen presenting cell as a part of a complex with a specific self-molecule encoded by a histocompatibility gene. The most potent histocompatibility genes are known as the major histocompatibility complex (MHC). The complex which is recognized by T-cell receptors, therefore, consists of and MHC/peptide ligand.

The ability to produce a soluble T-cell receptor which retains specificity for an antigenic peptide presented by an antigen presenting cell as a component of a complex including the MHC is useful in a variety of

contexts which are discussed in greater detail below. Several approaches have been employed to produce soluble, recombinant T-cell receptors. In these recombinant T-cell receptor molecules, the transmembrane/cytoplasmic regions of the α and β chains were replaced with sequences from lipid-linked proteins (GPI anchor), the CD3-ζ chain or with immunoglobulin (Ig) heavy or light chains. Soluble T-cell receptors were recovered either as secreted proteins (Ig constructs) or obtained by enzymatic cleavage of the GPI-anchor or the CD3-ζ chain. All of these approaches rely, however, on the assembly of the heterodimer which is inefficient. In addition, high level expression of the human T-cell receptor α chain in transfected eukaryotic cells is not stable.

These problems can be avoided by the design of a single chain recombinant protein in which the variable regions of the heterodimer are joined by a short peptide linker. Such a design has been successfully applied to antibody molecules. Such recombinant molecules, known as single-chain Fv's, have a specificity similar to that of native antibodies. Several groups have reported the production of single chain T-cell receptors in bacterial expression systems using the single chain antibody (Fv) design. However, due to the lack of functional data from these reports, it is unknown and impossible to predict whether the single chain Fv design yields soluble T-cell receptors that recognize the MHC/peptide ligand.

Summary of the Invention

The present invention relates in one aspect to a single chain T-cell receptor which binds specifically to an MHC/peptide ligand. The single chain T-cell receptor of the present invention has been demonstrated to be soluble in aqueous media. In a preferred embodiment, the single chain T-cell receptor is a 3-domain single

chain T-cell receptor comprising an α chain variable domain (Vα), a β chain variable domain (Vβ) and a constant domain. Specifically disclosed is a single chain T-cell receptor wherein the Vα-Jα domain of the α chain variable domain is linked to the N-terminus of the β chain variable domain by a flexible amino acid linker and the C-terminal region of the β chain variable domain is linked to the β chain constant domain (Cβ).

In another aspect, the invention relates to a self-signaling single chain T-cell receptor which binds specifically to an MHC/peptide ligand. The self-signaling T-cell receptor is preferably comprised of: 1) a soluble T-cell receptor domain comprising an α chain variable domain, a β chain variable domain and a constant domain; and 2) a transmembrane and intracellular signaling domain from a transmembrane receptor. When displayed on the surface of a T-cell, the self-signaling T-cell receptor has the ability to transduce signal following specific binding to MHC/peptide ligand.

The invention also relates to nucleic acid molecules which encode the single chain T-cell receptors described above, expression vectors containing such nucleic acid molecules, and cells which carry expressible copies of such nucleic acid molecules. In addition, the invention relates to methods for diagnosing viral infection in a patient. Disclosed are methods which employ both the single chain T-cell receptor, and the self-signaling embodiment of the single chain T-cell receptor.

Brief Description of the Drawing

Figure 1 is a diagrammatic representation of T-cell receptor α- and β-chain genes and various chimeric constructs which employs the following designations: S—S, disulfide bond; L, leader; V, variable segment; J, joining segment; C, constant region; TM, transmembrane

region; Cy, cytoplasmic region; ATG, start codon; Li, 15-residue peptide linker containing three repeats of GGGGS; PI, GPI domain of human placental alkaline phosphatase with the sequence LAPPAGTTDAAHPGRSVVPALLPLLAGTLLLL (SEQ ID NO. 3). The ζ region contains transmembrane and cytoplasmic domains of the marine CD3 ζ chain starting at position 31 (Engel et al., *Science 256*: 1318 (1992)).

Detailed Description of the Invention

The present invention is based on experiments which resulted in the synthesis of a single chain T-cell receptor which binds specifically to a predetermined MHC/peptide ligand which is presented by an antigen presenting cell (e.g, a macrophage). The MHC component of the MHC/peptide ligand can be Class I or Class II. The term "single chain" is used to describe an amino acid copolymer having a single N-terminus and a single C-terminus. This single chain T-cell receptor is to be contrasted with the native T-cell receptor which is comprised of two glycoprotein subunits which are referred to as the α and β chains. Another distinction between the single chain T-cell receptor of the present invention, and the native T-cell receptor, is that the single chain T-cell receptor of the present invention is soluble in an aqueous medium. The term "aqueous medium", as used herein, includes, for example, physiologically compatible buffered solutions. It has been determined, for example, that the single chain T-cell receptor of the present invention is soluble in phosphate buffered saline up to a concentration of 1 mg/ml.

As discussed above, the native T-cell receptor is comprised of two glycoprotein subunits which are referred to as the α and β chains. Each of the glycoprotein subunit chains comprise two domains, one variable and one constant. Like antibodies, the

glycoprotein subunits are encoded by genes which are formed by rearrangement of segments (V, D and J segments (β chain), and V and J segments (α chain)) from libraries found in discrete chromosomal regions.

In a preferred embodiment of the present invention, the single chain T-cell receptor is a 3-domain single chain T-cell receptor comprising a leader sequence and an α chain variable domain, a β chain variable domain and a constant domain. While the order of these domains within the single chain T-cell receptor may be interchangeable, the preferred ordering of the domains is specified below. Such a molecule can be prepared in a variety of ways. For example, conventional synthetic techniques may be employed to produce the desired amino acid copolymer from its component amino acids (see e.g., Houghton et al., *Proc. Natl. Acad. Sci. USA 82*: 5135 (1985)).

Preferably, DNA encoding the single chain T-cell receptor is generated at the genetic level by combining elements from isolated T-cell receptor genes by recombinant DNA techniques (see e.g., Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982)). In order to practice such methods it is first necessary to isolate nucleic acid which encodes the α and β chains of the T-cell receptor having the desired specificity. The generation of DNA encoding the α and β chains which comprise a T-cell receptor which binds specifically with a predetermined epitope of the human myelin basic protein (MBP), when presented in association with an MHC Class II protein, is described in detail in the Examples which follow. The procedures specified in connection with the isolation of DNA encoding a T-cell receptor specific for an epitope of the human MBP are applicable to the isolation of T-cell receptor genes which encode a T-cell receptor which binds specifically to any predetermined MHC/peptide ligand.

Briefly, an initial step in obtaining DNA encoding T-cell receptor genes which encode a T-cell receptor which binds specifically to any predetermined MHC/peptide ligand is to isolate an antigen specific T-cell clone from a mammal (preferably a human) which is known to possess the antigen of interest in its tissue. For example, if the predetermined MHC/peptide ligand contains a peptide representing a T-cell epitope from an HIV virus, an individual infected with the virus would be selected.

A peripheral blood cell preparation is obtained from the selected individual by conventional techniques (Wucherpfennig et al., *Science 248*: 1018 (1990); Wucherpfennig et al., *J. Immunol. 152*: 5581 (1989)). The peripheral blood cell population is stimulated by contacting the cells with the predetermined antigen using conventional techniques intended to expand the population of T-cells which bind specifically to the antigen of interest. Typically stimulation is carried out with a purified peptide known to contain a T-cell epitope, or with an enzymatically degraded protein preparation (e.g., a partial trypsin digest) comprising a mixture of peptides. This stimulation procedure enriches the T-cell population for those T-cells which specifically bind to an epitope of the antigen of interest. Through dilution, expansion and binding studies, a clonal population of cells encoding a single T-cell receptor specific for the antigen of interest is isolated.

DNA encoding the α and β chains which comprise a T-cell receptor is then prepared from the clonal T-cell population using spliced mRNA as template. One way in which this can be accomplished is through the construction of a cDNA library from a clonal cell culture of the type described above. The isolation of mRNA and the synthesis and cloning of double stranded cDNA molecules encoded by the mRNA is a routine

procedure (see e.g., Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982)). The cDNA library is then screened using probes specific for the constant region of the T-cell receptor α or β chains (see e.g., Davis et al., *Nature 334*: 395 (1988)).

A variety of alternative approaches can also be used to prepare DNA encoding the α or β chains from mRNA isolated from a cell culture demonstrating the desired binding specificity. Such methods include the use of a standard PCR protocol using first-strand cDNA template prepared from the isolated mRNA. The latter method is, in fact, the method which was used to generate DNA encoding the β chain in the experiments described in detail in Example 1 which follows. Yet another alternative is the anchored PCR technique described by (Loh et al., *Science 243*: 217 (1989)). Using standard recombinant DNA techniques, a 3-part single chain T-cell receptor of the type described herein can be assembled from domains isolated from the α and β chain DNA.

A leader sequence must be included for proper intracellular processing of the single chain T-cell receptor. In genomic DNA, a leader sequence is always linked to the DNA encoding the α chain variable domain such that in the encoded product, the leader sequence is located at the N-terminus. Therefore, in the T-cell receptor of the present invention, a leader sequence is located at the N-terminus of the molecule and is considered to be an element of the Vα domain. It will be recognized, however, that although a specific leader sequence is linked to a specific Vα domain in genomic DNA, leader sequences can be exchanged using recombinant DNA techniques without a detrimental affect on the intracellular processing of the encoded fusion. Thus, in the T-cell receptor of the present invention, the Vα-Jα domain of the α chain variable domain is linked to C-terminus of a leader sequence.

The Vα-Jα domain of the α chain variable domain is also linked to the N-terminus of the β chain variable domain by a flexible amino acid linker and the C-terminal region of the β chain variable domain is linked to the β chain constant domain. In addition to these domains which must be present at a minimum, it may be possible to add additional segments without interfering with the essential properties of the encoded molecule.

The identity of individual amino acids in the amino acid linker, and the number of amino acids which comprise the amino acid linker are variable. The purpose of this linker is to provide sufficient flexibility within the molecule to enhance the binding characteristics of the soluble T-cell receptor. Typically a linker comprised of about 10 to 30 amino acid residues would be considered sufficient. With regard to the identity of the amino acids in the linker region, glycine is preferred in light of the fact that a glycine polymer exhibits increased flexibility relative to other mono-amino acid polymers due to the fact that it lacks β-carbons. In addition, amino acids which tend to increase solubility in an aqueous solution are preferred (e.g., serine, glutamine, aspartic acid, arginine, etc.).

It will be recognized by those skilled in the art that conservative amino acid substitutions introduced (e.g., by site directed mutagenesis) within the constant region may be tolerated without adversely affecting the binding specificity of the T-cell receptor. Furthermore, the framework of a T-cell receptor variable region (i.e., the portion of the variable region other than the CDRs) can be exchanged with corresponding variable regions from other T-cell receptors without adversely affecting the specificity of the receptor.

In addition, it will also be recognized that such conservative substitutions may be tolerated within the variable regions so long at they do not occur within a

complimentarity determining region (CDR), which is sometimes referred to as the hypervariable region. In addition, the scope of the present invention is intended to encompass derivatives of the T-cell receptor of the invention wherein the addition of the derivatizing group would not be expected to substantially alter the binding characteristics of the soluble T-cell receptor. Such modifications include, for example, the addition of a radiolabel group or the conjugation of a toxin molecule. The term "conservative substitution", as used herein, refers to the substitution of a second amino acid residue for a first amino acid residue, both residues having an R group exhibiting substantially similar chemical properties. Standard biochemistry textbooks provide groupings of amino acid residues according to substantial similarity in R group chemistry.

DNA encoding the single chain T-cell receptor, produced in the manner described above, can be expressed in eukaryotic or prokaryotic cells by placing the DNA under the control of regulatory sequences appropriate for expression in the cell type of interest, and introducing the construct into the cell type of interest. Regulatory sequences appropriate for expression can exert their influence at many levels including, for example, the transcriptional, translational and post-translational levels. The DNA encoding the single chain T-cell receptor, together with appropriate regulatory sequences are typically carried within a larger DNA molecule (often referred to as an expression construct) which facilitates the introduction and expression of the expressible nucleic acid encoding the single chain T-cell receptor. An extrachromosomal plasmid is an example of an expression vector which could be used to facilitate prokaryotic expression. Modified eukaryotic viral DNA capable of integrating into the chromosome of a host-cell is an example of an expression vector which could be used to facilitate eukaryotic expression. The selection of expression

vectors and appropriate regulatory signals is highly dependent upon the particular application. Given a particular experimental goal, the choice of an appropriate vector and appropriate regulatory sequences would be clear to one of skill in the art based on fundamental and well established principles.

In another embodiment, the present invention relates to a self-signaling single chain T-cell receptor which binds specifically to an MHC/peptide ligand when presented by an antigen presenting cell. The native T-cell receptor is not a self-signaling molecule. Rather, the signaling function of the native T-cell receptor is mediated by a number of ancillary proteins. Briefly, there are two major classes of T-cells: cytotoxic T lymphocytes (CTLs) and helper T (T_H) lymphocytes. CTLs have the ability to lyse cells which display a foreign antigen on their cell surface (in association with an MHC component). T_H lymphocytes, on the other hand, recognize the degradation products of foreign antigens and, in response, secrete stimulatory factors which induce response by other immune system components. CTLs can be distinguished from T_H lymphocytes based on the presence of certain surface proteins. More specifically, CTLs typically have the CD8 protein on their cell surface while T_H lymphocytes typically have CD4 protein on their cell surface.

The interaction between the antigenic peptide/MHC complex on an antigen presenting cell, and the T-cell receptor on the T-cell, is thought to involve either a class I MHC-CD8 interaction or a class II MHC-CD4 interaction. A variety of other proteins also participate in the effector signaling process which results ultimately in the secretion of protein factors by both classes of T-cells (the secreted protein factors include, for example, factors which induce cell lysis in the case of CTLs, and lymphokines in the case of T_H lymphocytes). Other proteins which participate in the signaling process include, for example, the CD3 complex

of proteins which are tightly bound to the T-cell receptor, as well as a tyrosine kinase protein encoded by the *lck* gene which is bound to both CD4 and CD8. In summary, the specific interaction of an antigenic peptide/MHC complex with a native T-cell receptor triggers a complex signaling process involving multiple protein participants which results ultimately in the secretion of certain protein factors which play a role in the immune response.

The self-signaling T-cell receptor of the present invention differs from the native form in that a signaling domain is incorporated as a component of the fusion protein. More specifically, the self-signaling T-cell receptor of the present invention includes all of the elements of the single chain T-cell receptor described above (i.e., an α chain variable domain, a β chain variable domain and a constant domain) as well as a transmembrane and intracellular signaling domain from a transmembrane receptor. As discussed previously, a leader sequence is essential for intracellular processing and is located at the N-terminus of the molecule. The C-terminus of the leader sequence is linked to the N-terminus of the Vα-Jα domain of the α chain variable domain. This, in turn is linked at its C-terminus to the N-terminus of the β chain variable domain by a flexible amino acid linker. The C-terminal region of the β chain variable domain is linked to the β chain constant domain and the C-terminal region of the β chain constant region is linked to the N-terminal region of the transmembrane and intracellular signaling domain from a transmembrane receptor. As was discussed above in connection with the non-signaling single chain T-cell receptor, construction of the self-signaling single chain T-cell receptor is preferably carried out at the genetic level although alternative methods are well known in the art. DNA encoding the transmembrane/signaling domain of the transmembrane

receptor is also isolated according to well established experimental steps protocols including, for example, standard PCR.

The identity of the transmembrane receptor from which the transmembrane and intracellular signaling domain is derived depends upon the nature of the signal which is preferred. For example, if it is desired that self-signaling T-cell receptor is to be displayed on the surface of a T-cell with the ultimate goal being the stimulation of a response which mimics the T-cell response in vivo (e.g., secretion of protein factors such as lymphokines (T_H lymphocytes) or factors which induce cell lysis (CTLs)), the preferred transmembrane and intracellular domains are derived from the CD3 ζ chain. A more detailed discussion of the use of this signaling domain in a specific experimental context is provided in Example 1 which follows. In addition, other signalling domains such as the transmembrane and intracellular domains of Fc_γ are also useful in this context to produce a response which mimics the response of a stimulated T-cell (see e.g., Eshhar et al., *Proc. Nat. Acad. Sci. USA 90*: 720 (1993)). The ability to generate a T-cell clone having: 1) a predetermined MHC/peptide ligand specificity; and 2) the ability to mimic activity of cytotoxic T lymphocyte following binding to the MHC/peptide ligand offers the opportunity for a new method of therapeutic intervention which is discussed in greater detail below.

If, on the other hand, a T-cell response which mimics the natural response of a stimulated T-cell is not required, a great variety of transmembrane/signaling domains can be used. Based on previous work with chimeric transmembrane receptors, it would be predicted with a high degree of certainty that the fusion of the extracellular domain of the single chain T-cell receptor of this invention, with the transmembrane/signaling domain of virtually any transmembrane receptor, would

result in the production of a chimeric single chain T-cell receptor in which the signaling function would be operable following specific binding of the extracellular domain of the single chain T-cell receptor to the MHC/peptide ligand for which it is specific.

For example, the intracellular and transmembrane domains of a tyrosine kinase receptor can be used. Many transmembrane tyrosine kinase receptors have been identified (for reviews see, e.g., Hanks, *Current Opinion in Structural Biology 1*: 369 (1991) and Pawson and Bernstein, *Trends in Genetics 6*: 350 (1990)). Such a receptor, when stimulated, functions to phosphorylate intracellular protein targets. In this instance, an increase in intracellular phosphorylation could be detected using specific antibodies (i.e., anti-phosphotyrosine antibodies such as antibody 4G10 which is available commercially from Upstate Biotechnology Incorporated) thereby providing a positive indication of specific binding of the self-signaling T-cell receptor to its MHC/peptide ligand binding partner. Such a detection method would be useful, for example, in connection with a diagnostic embodiment of the present invention which is described below.

Preferably the self-signaling single chain T-cell receptor is expressed in a T lymphocyte which is then clonally expanded. In theory, any T lymphocyte can be employed as a host-cell for expression of the self-signaling T-cell receptor. However, for many applications, expression in a cytotoxic T lymphocyte is preferred. T lymphocytes to be used as host-cells can be purified from the peripheral blood cells of an individual. Alternatively, established T lymphocyte tumor cell lines can be employed. Many such tumor cell lines have been reported in the literature. Expression is accomplished by the introduction of an expressible genetic construct carrying DNA encoding the self-signaling single chain T-cell receptor into the T lymphocyte. As discussed previously, the ability to

design and synthesize such an expressible genetic construct is a matter of routine experimentation given the current state of the art.

The compositions described above are useful in a variety of contexts. For example, the single chain T-cell receptor and related compositions are useful in a method for diagnosing viral infection in a patient. In this method of diagnosis, a single chain T-cell receptor which binds specifically to an antigenic peptide from the virus when presented by an antigen presenting cell in association with an MHC is produced as described previously. To facilitate detection, the soluble T-cell receptor may be labeled with a reporter group (e.g., a radiolabeling group).

A peripheral blood cell preparation from the individual to be tested for viral infection is then incubated with the soluble single chain T-cell receptor under conditions appropriate for binding of the soluble single chain T-cell receptor to the MHC/peptide ligand for which it is specific. Such conditions include, for example, incubation in a physiologically compatible buffer at a temperature of about 4° C. Following an appropriate incubation period (e.g., 30 minutes) the cells are pelleted and resuspended in a wash solution to remove labeled single chain T-cell receptor which is not specifically complexed with an MHC/peptide ligand on the surface of antigen presenting cells.

Following the removal of non-specifically bound receptor, the amount of radiolabel associated with the cellular fraction is determined. This determination can be made using a variety of standard techniques depending upon the identity of the label employed. Values determined in this manner are then compared with values determined in an otherwise identical incubation using a peripheral blood cell preparation from an individual known to be noninfected with the virus of interest. Viral infection is indicated by the detection of substantially greater signal (i.e., at least about 2-

fold) from the peripheral blood cell preparation from the individual to be tested for viral infection when compared with the signal detected using the peripheral blood cell preparation from the individual known to be noninfected.

The method of diagnosing viral infection discussed above can also be modified to employ a self-signaling T-cell receptor of the type described above. In this method of diagnosis it is not necessary that the signal produced by receptor binding to the MHC/peptide ligand result in a T-cell response which mimics the T-cell response in vivo (i.e., secretion of protein factors). Rather, all that is required is that specific binding of the self-signaling single chain T-cell receptor to the MHC/peptide ligand result in the generation of a specific and detectable signal. In light of this, the transmembrane/signaling domain of the self-signaling T-cell receptor can be selected from virtually any transmembrane receptor possessing both a transmembrane and a signaling domain. It will be recognized, of course, that the transmembrane/signaling domain from a transmembrane receptor known to participate in a signaling process which stimulates a T-cell response which mimics the T-cell response in vivo can be used. An example of such a transmembrane receptor is the CD3 ζ-chain.

DNA encoding the self-signaling single chain T-cell receptor is introduced in expressible form into a mammalian T-cell. When incubated under conditions appropriate for metabolic activity, the self-signaling single chain T-cell receptor is expressed and displayed on the surface of the host-cell.

A peripheral blood cell preparation from an individual to be tested for infection is provided. The peripheral blood cell preparation is subsequently fixed in solution using a fixing agent (e.g., paraformaldehyde). In addition to providing other

experimental advantages, fixing the antigen presenting cell ensures that any cytokine secretion subsequently detected is secreted from the T-cell. Following a wash step to remove excess fixing agent, the fixed cells and the cells bearing the self-signaling single chain T-cell receptor are incubated together under conditions appropriate for binding of a T-cell receptor to the MHC/peptide target for which it is specific.

Specific binding of the self-signaling single chain T-cell receptor to the MHC/peptide ligand for which it is specific results in a triggering of the signaling domain of the self-signaling receptor. Signal is then detected by conventional techniques. For example, if the signaling domain is from a tyrosine kinase receptor, signal is detected using anti-phosphotyrosine antibodies. If, on the other hand the signaling domain is from a transmembrane receptor having the properties CD3 ζ-chain, signal is detected for example by the detection of secreted lymphokine (e.g., IL-2) using appropriate immunological reagents. The level of signal determined is then compared with levels determined in an otherwise identical experiment using a peripheral blood cell preparation from an individual known to be noninfected.

In addition to the diagnostic applications discussed above, the soluble single chain T-cell receptor of the present invention (and the self-signaling counterpart) can be used in a therapeutic context. Soluble T-cell receptors with proper specificity, either alone or with another functional group (such as toxins) can be used to block specific immune response or target malignant or virus-infected cells. The self-signaling single chain T-cell receptor, when expressed in a suitable host-cell, can be applied to regulate specific immune response or to target malignant or virus-infected cells.

The single chain design of the T-cell receptors of this invention will also facilitate the construction of T-cell receptor phage libraries similar to those made for single chain antibodies (Clackson et al, *Nature 352*: 624 (1992)). The success in producing a bacterially-expressed three-domain single chain design supports this application. Such libraries are powerful tools for the isolation of T-cell receptors with defined specificity and/or high affinity.

EXAMPLE 1

The present example discloses studies in which different single chain T-cell receptor designs were evaluated in transfected eukaryotic cells with respect to surface expression of T-cell receptor molecules, proper folding and recognition of the appropriate MHC/peptide ligand. A single-chain three-domain construct (Vα-linker-Vβ-Cβ) was stably expressed on the cell surface when linked to a glycosyl-phosphatidylinostol (GPI) anchor and recognized by a conformation dependent mAb specific for the Vβ17 segment. The soluble form of this recombinant protein could be readily obtained by enzymatic cleavage with phosphatidylinostol-specific phospholipase C (PI-PLC). Replacement of the GPI domain with the cytoplasmic portion of the ζ-chain resulted in a functional T-cell receptor molecule that transduced an intracellular signal following recognition of the proper MHC/peptide ligand and the superantigen staphylococcal enterotoxin B (SEB).

cDNAs of T-cell receptor α and β chains were prepared from mRNA of 2H9 cells with Superscript reverse transcriptase (BRL) and an oligo(dT) primer (Sigma) and were amplified by PCR using Vent DNA polymerase (New England Biolabs) and primers 5′-GCTCGAGGCGGCGATGGAAACTCTCCTGGGAGT-3′ (A5) (SEQ ID NO. 4)

and 5′-GGAATTCAGCTGGACCACAGCCGC-3′ (SEQ ID NO. 5) for α chain and 5′-GCTCGAGCTCTGCCATGGACTCCTGGA-3′ (SEQ ID NO. 6) and 5′-GGAATTCAGAAATCCTTTCTCTTGAC-3′ (SEQ ID NO. 7) for β chain. The cDNAs were cloned into the mammalian expression vector pBJ-neo (Engel et al., *Science 256*: 1318 (1992)). GPI-anchored T-cell receptor molecules (α-PI and β-PI) were constructed as follows. A *Ban* I site was engineered after the fifth amino acid residue beyond the last cysteine by oligonucleotide-directed mutagenesis. The region 3′ of the *Ban* I site was then replaced with a *Ban* I-*Not* I fragment encoding the GPI signal domain from the human placental alkaline phosphatase. For the construction of various single chain T-cell receptors, variable domains of the 2H9 T-cell receptor α and β chains were prepared by PCR using primers A5 and 5′-CAGAGCTCACGGATGAACAATAAGGCTGGT-3′ (SEQ ID NO. 8) for the Vα domain in all the single chain T-cell receptor constructs, 5′-TCGGATATCGATGGTGGAATCACTCAGTCC-3′ (B5) (SEQ ID NO. 9) and 5′-CAGAGATCAGCACGGTGAGCCGGTTCCC-3′ (SEQ ID NO. 10) for the Vβ domain in AB-PI-1, 5′GTGGGAGATCTCTGCTTCTGATGGCTCAAAC (SEQ ID NO. 11) and B5 for the Vβ domain in AB-PI-2, 5′-CACGGATCCCCGTCTGCTCTACCCCAGGC (SEQ ID NO. 12) and B5 for the Vβ and Cβ domains in ABC-PI, and 5′-CACGGATCCCCGTCTGCTCTACCCCAGGC-3′ (SEQ ID NO. 13) and B5 for the Vβ and Cβ domains in ABC-ζ. The cDNA encoding the transmembrane and cytoplasmic domains of murine CD3 ζ chain (Engel et al., *Science 256*: 1318 (1992)) was employed. Convenient restriction sites were engineered at the end of each fragment to aid in the assembly of the construct. The linker was a 15-amino acid motif of GGGGS repeated three times (Huston et al., *Int. Rev. Immunol. 10*: 195 (1992)) with *Sac* I at the 5′ end and *Eco*RV at the 3′ end. Except for α-PI, all the constructs were cloned into pBJ-neo, which carries the G418-resistance gene. α-PI was cloned into pCEP-4

(Invitrogen), which bears the hygromycin-resistance gene. All constructs were verified by multiple restriction digests and by sequencing with the Sequenase kit (United States Biochemical).

Recombinant T-cell receptor molecules were generated by using the T-cell receptor α- and β-chain sequences of the human myelin basic protein (MBP) specific T-cell clone Hy.2H9 (Wucherpfennig et al., *Science 248*: 1016 (1990); Wucherpfennig et al., *J. Immunol. 152*: 5581 (1994)). This clone T-cell receptor is composed of the Vα3.1 and Vβ17.1 segments and is specific for the immunodominant MBP peptide MBP-(85-99) in the context of HLA-DR2 (DRA, DRB1*1602) (Wucherpfennig et al., *J. Immunol. 152*: 5581 (1994)). Usage of the Vβ17.1 segment allowed the proper folding of recombinant T-cell receptors to be probed using the superantigen SEB (Choi et al., *Proc. Natl. Acad. Sci. USA 86*: 8941 (1989)) and the monoclonal antibody C1 (Friedman et al., *J. Exp. Med. 174*: 891 (1991)). To confirm the Vβ17 specificity of C1, the extracellular domains of T-cell receptor α and β chains of Hy.2H9 cells were fused to the C-terminal sequence from human placental alkaline phosphatase for GPI anchorage (Fig. 1) and the DNAs encoding the GPI-anchored β and α chains (β-PI and α-PI) were sequentially transfected by electroporation into a T-cell receptor α- and β-chain-deficient murine lymphoma cell line, BW5147 $\alpha^-\beta^-$ (BW^-) (White et al., *J. Immunol. 143*: 1822 (1989)). The surface expression of the GPI-anchored T-cell receptor chains was monitored by staining with mAbs αF1 (Henry, et al., *Hybridoma 8*: 577 (1990)), βF1 (Brenner et al., *J. Immunol. 138*: 1502 (1987)), and C1. αF1 and βF1 recognize nonconformational epitopes located in the C region of the T-cell receptor α and β chains, respectively. Surface expression of the T-cell receptor β chain is independent of heterodimer formation and assembly of the CD3 complex (Lin et al., *Science 249*:

677 (1990)). In the β-PI-transfected cells, high-level expression of β-PI was confirmed by staining with βF1. Interestingly, there was little C1 staining of these transfectants. However, when a GPI-anchored 2H9 α chain was supertransfected into the β-PI transfectant, C1 reactivity was greatly increased while the level of βF1 staining remained constant. Thus, the Vβ17-specific C1 epitope is conformational and dependent on the proper pairing of T-cell receptor α and β chains and can therefore be used to assess the proper folding of recombinant T-cell receptors bearing a Vβ17 sequence.

High-level expression of the T-cell receptor α chain (α-PI) was, however, not stable either alone or in the presence of β-PI. Attempts were made on several cell lines, including COS-7, CHO-K1, and a T-cell receptor-deficient variant of Jurkat-cells, JK-β⁻ (J.RT3-T3.5, American Type Culture Collection). The expression level of α-PI was comparable to that of β-PI after the initial drug selection, but continued culture for less than a month yielded a population of cells with little surface express of α-PI, whereas β-PI expression was stable. The inability to obtain cell lines with stable high-level expression of the PI-anchored human T-cell receptor α chain has been reported previously (Devaux et al., *Eur. J. Immunol. 21*: 2111 (1991)).

To overcome the limitation set by the unstable expression of the human T-cell receptor α chain, various sc designs were examined. Initially, a design similar to that of sc antibodies (Fv) was chosen (Huston et al., *Int. Rev. Immunol. 10*: 195 (1992)). A 15-residue flexible linker was used to link the C terminus of the Vα-Jα domain to the N terminus of the β chain. The GPI domain was then ligated to the C terminus of the Vβ-Jβ domain. The construct (AB-PI-1, Fig. 1) was transfected into several cell lines, including JK-β⁻, COS-7, CHO-K1, and BW⁻. Although the expression of the gene was confirmed by the detection of the correct RNA

transcripts, no surface expression was detected, as evidenced by negative C1 antibody staining. Immunoprecipitation after metabolic labeling failed to recover any C1-reactive sc molecules from these transfectants. The inability to identify any C1-reactive protein could have been due to the design of this molecule, such as insufficient linker length between the extracellular domain and the GPI domain. To improve the accessibility of the sc construct, another two-domain single chain T-cell receptor was designed in which an extra 30-amino acid portion of the N terminus of the Cβ domain was added as a hinge region. The transfectants of this construct (AB-PI-2, Fig. 1) were still not reactive with the C1 antibody. Finally, the entire Cβ domain was added to the sc construct. A complete Cβ domain should provide enough distance for the Vα-Vβ domains to be expressed on the cell surface and, more importantly, should allow surface expression to be monitored with another antibody, βF1 (Brenner et al., *J. Immunol. 138*: 1502 (1987)). This three-domain sc T-cell receptor was constructed by extending the T-cell receptor β-chain sequences to the residue right before the last cysteine (the sixth cysteine), which was then fused to the GPI domain. The last cysteine was deleted to prevent dimerization between Cβ domains. This construct (ABC-PI, Fig. 1) was transfected into BW cells and surface expression was confirmed by staining with both βF1 and C1. Both antibodies stained the cells with comparable efficiency, suggesting that most of the molecules were expressed in the correct conformation. Moreover, the molecule could be efficiently cleaved from the cell surface with PI-PLC.

Soluble three-domain single chain T-cell receptor was purified from transfectants after PI-PLC cleavage followed by affinity chromatography using the βF1 antibody. More specifically, after transfection and G418 selection (Engel et al. *Science 256*: 1318 (1992)),

cells expressing a high level of GPI-linked three-domain single chain T-cell receptor (ABC-PI) were isolated by three rounds of sorting. The resulting cells were grown in spinner culture to a density of 10^6 per ml and harvested by centrifugation. The pellet was washed twice with phosphate-buffered saline (PBS) and resuspended in PBS containing 2 mM Pefabloc protease inhibitor (•••) (Centerchen, Inc., Stamford, CT) to a density of 5×10^7 per ml with PI-PLC (Sigma) added at 1 unit/ml. Cells were incubated at 37°C for 1 hr with constant rocking. The supernatant was collected by centrifugation and by passage through a 0.45-μm filter and applied to a column of Acti-gel (•••) (Sterogen, Inc., Arcadia, CA) with immobilized βF1. The column was washed with 10 volumes of PBS and the soluble T-cell receptor was eluted with 0.15 M glycine (pH 2.8). Fractions were immediately neutralized with 0.1 volume of saturated Tris. The soluble T-cell receptor was then dialyzed against > 100 volumes of PBS at 4°C with at least four changes and concentrated to 0.5 mg/ml by vacuum dialysis against PBS. Five micrograms of purified soluble three-domain single chain T-cell receptor was analyzed by SDS/PAGE under reducing conditions.

The purified three-domain single chain T-cell receptor appeared as multiple bands at 50-70 kDa after SDS/PAGE. The observed heterogeneity of single chain T-cell receptor is probably the result of variable glycosylation; its polypeptide size calculated from amino acid composition is 40 kDa. The structural integrity of the three-domain single chain T-cell receptor was verified by a two-antibody ELISA. The molecules were first captured by the βF1 antibody immobilized to the plate and then assessed for reactivity with the C1 antibody. When compared with the three-domain single chain T-cell receptor produced in a bacterial expression system, the single chain T-cell

receptor from the eukaryotic system gave 10-20 times higher C1 reactivity. The purified three-domain single chain T-cell receptor was stable and could be stored in PBS at 4°C for months without significant loss of C1 reactivity.

To directly assess the functional integrity of the three-domain single chain T-cell receptor, a self-signaling single chain T-cell receptor was produced by replacing the GPI domain with the transmembrane and cytoplasmic domains of the CD3 ζ chain. These regions have been shown to be sufficient for signal transduction when its extracellular fusion partner is crosslinked by an antibody or by the proper ligand (Engel et al. *Science 256*: 1318 (1992); Irving et al., *Cell 64*: 891 (1991); Romeo et al., *Cell 64*: 1037 (1991)). To enable the recovery of three-domain single chain T-cell receptor as a soluble form, a linker containing a thrombin cleavage site was inserted into the junction of three-domain single chain T-cell receptor and the ζ domain. The construct (ABC-ζ) was transfected into BW^- cells and the rat basophilic leukemia cell line RBL-2H3 (RBL) (Engel et al. *Science 256*: 1318 (1992)), and the populations displaying high-level expression of three-domain single chain T-cell receptor were isolated by three rounds of cytofluorometric sorting using the antibody βF1. The ABC-ζ-transfected cells were first stimulated with various antibodies to confirm the self-signaling nature of this recombinant molecule.

More specifically, ABC-ζ-transfected BW5147β $\alpha^-\beta^-$ cells (5×10^4 per well) were cultured in a 96-well round-bottom plate to which various antibodies had been immobilized (1 μg per well). The supernatants were collected after 24 hr and interleukin 2 (IL-2) production was assessed in a bioassay using an IL-2-dependent-cell line (CTLL) and the CellTiter-96 nonradioactive proliferation assay (Promega). In the

case of ABC-ζ-transfected RBL-2H3 (Engel et al., *Science 256*: 1318 (1992)) cells, the cells were incubated with [^{3}H]serotonin (NEN) at 0.5 μCi (18.5 kBq) for 24 hr before they were added to the antibody plate. After incubation at 37°C for 2 hr, radioactivity released into the supernatant was measured in a liquid scintillation counter. The specific serotonin release was calculated as described (Engel et al., *Science 256*: 1318 (1992)). For SEB stimulation, 5 X 10^4 transfected cells per well were cultured with various concentrations of SEB (Toxin Technology, Madison, WI) in the presence or absence of 2 X 10^5 B cells. For antigen presentation, 5 X 10^4 transfected cells per well were cocultured with 2 X 10^5 B cells which were incubated with or without the myelin basic protein (MBP)-(85-89) peptide for 3 hr before the experiment. The assays were conducted as described above.

Both transfectants showed a strong response following βF1 and C1 stimulation but not to purified mouse immunoglobulin or anti-CD8 antibody used as controls. The structural integrity of the single chain T-cell receptor was further examined with the superantigen SEB, which binds to both Vβ17 and MHC class II molecules, resulting in T-cell receptor crosslinking and T-cell activation regardless of the peptide bound to the MHC molecule (Choi et al., *Proc. Natl. Acad. Sci. USA 86*: 8941 (1989); Irwin et al., *Immunol. Rev. 131*: 61 (1993)). ABC-ζ transfectants displayed a concentration-dependent response toward SEB when the superantigen was presented by transformed B-cell lines with high-level expression of DR1 (DRA, DRB1*0101; cell line LG2) or DR2 (DRA, DRB1*1602; cell line 9016). Thus, the lateral face of the T-cell receptor Vβ region to which SEB is thought to bind (Choi et al., *Nature (London) 346*: 471 (1990)) is structurally intact.

To prove that the three-domain single chain T-cell receptor did indeed recognize the MHC/peptide ligand, antigen presentation experiments using the natural ligand for the 2H9 clone, 9016 cells bearing the DRB1*1602 allele of DR2, and MBP-(85-99) peptide were performed. To ensure detection of subtle abnormalities in the structure of the three-domain single chain T-cell receptor, 9009 cells (DRA, DRB1*1601), which also bind MBP-(85-89), were used as a control. DRB1*1601 and DRB1*1602 differ only at position 67 in the DRβ1 domain; this T-cell receptor contact-residue substitution does, however, abolish recognition of the peptide by the parent T-cell clone (Wucherpfennig, et al, *J. Immunol. 152*: 5581 (1994)). The ABC-ζ transfected BW$^-$ cells secreted IL-2 in response to peptide-pulsed 9016 cells, but not to peptide-pulsed 9009 cells. Similar results were obtained with RBL transfectants, as serotonin release was dependent on the concentration of the MBP peptide used to pulse 9016 cells. The signal appeared to be weak when compared with antibody stimulation. This is not surprising, however, since saturating amounts of antibodies are expected to crosslink the majority of T-cell receptor molecules on the target-cell, whereas a much smaller fraction of T-cell receptor molecules is probably engaged when T-cells are cocultured with peptide-pulsed antigen-presenting cells, in which a maximum of 5-15% of the DR molecules bind the peptide. The requirement for a high concentration of peptide or SEB is not due to the sc design, since high concentrations of peptide are also needed to stimulate $\alpha\beta$ heterodimers of the T-cell receptor-ζ constructs (Engel et al. *Science 256*: 1318 (1992)). It is likely that the decrease in sensitivity results from the lack of CD3, CD4, and/or other adhesion/signaling molecules. Nonetheless these results demonstrate that the three-domain single chain T-cell receptor was correctly folded and functionally competent. In addition, a soluble form

of three-domain single chain T-cell receptor could be obtained from the ABC-ζ transfectants by thrombin cleavage and affinity purification.

EXAMPLE 2

Materials and Methods

i) Construction of the single chain T-cell receptors

Recombinant T-cell receptor molecules were generated using the T-cell receptor α and β chain sequences of the human myelin basic protein (MBP) specific T-cell clone Hy.2H9 as described in Example 1. This T-cell receptor is composed of the Vα3.1 and Vβ17.1 segments and is specific for the immunodominant MBP(85-99) peptide in the context of HLA-DR2 (DRA, DRB1*1602). cDNAs for the T-cell receptor α and β chains of the Hy.2H9 cells were prepared as described in Example 1. DNA fragments corresponding to the variable or constant domains of the α and β chains were prepared by PCR using cDNA template with convenient restriction sites engineered at the ends of each fragment. The α and β-chain domains were connected by a 15-residue peptide linker with the primary sequence of GGGGS GGGGS GGGGS (SEQ ID NO. 14) (Huston et al., *Proc. Natl. Acad. Sci. USA 85*: 5879 (1988)). The assembled single chain T-cell receptor was cloned in frame into a bacterial expression vector with PelB signal sequence and an isopropyl β-D-thiogalactopyronoside (IPTG) inducible Tac promoter. The nucleotide sequence of the final construct was confirmed using the Sequenase sequencing kit (USB) and the complete sequence is shown in SEQ ID NO: 1.

ii) Expression, purification and refolding of the single chain T-cell receptors

To induce expression of single chain T-cell receptors, E. coli strain BMH transformed with the single chain T-cell receptor plasmid was grown in Luria

broth containing 15 μg/ml tetracycline and 2% glucose (w/v) at 30°C to a density at A_{600} of 0.5 to 1.0. Cells were then pelleted by centrifugation and washed once with Luria broth. The cells were resuspended in fresh Luria broth containing 1 mM IPTG, grown for a further 8-12 hours at 37°C before being harvested by centrifugation. The cell pellet was resuspended in 10 ml/3g cell paste of 50 mM Tris-HCl (pH 8.0)/1 mM EDTA/lysozyme (1 mg/ml)/1 mM phenylmethylsulfonyl fluoride (PMSF) and incubated on ice for 30 min. DNase and $MgCl_2$ were added to final concentrations of 10 μg/ml and 10 mM respectively and the suspension was incubated for 30 min at room temperature. EDTA was added to a concentration of 20 mM and the suspension was subjected to 2 cycles of freeze/thaw. An additional 20 mM $MgCl_2$ was added to the suspension and incubated at room temperature for a further 15 min. After addition of EDTA to a final concentration of 40 mM, the cell suspension was centrifuged at 10,000 xg for 30 min at 4°C. The supernatant was removed and the cell pellet containing inclusion bodies was washed twice with 0.5% Triton x-100/50 mM Tris-HCl (pH 8.0)/100 mM NaCl/0.1% sodium azide. After a final wash in 50 mM Tris-HCl (pH 8.0)/100 mM NaCl, the pellet containing inclusion bodies was solubilized in 8M urea/100 mM $NaHPO_4$/10 mM Tris-HCl (urea buffer) (pH 8.0). The suspension was centrifuged at 10,000 xg for 30 min, and the supernatant was loaded onto a Ni-NTA column (Qiagen) and washed with 10 volumes of urea buffer (pH 8.0) and then 5 volumes of urea buffer (pH 6.3). Protein was eluted in fractions from the column in 2 volumes of urea buffer (pH 4.5). Protein content was determined by a Coomassie blue protein assay, and fractions containing proteins were pooled and diluted in refolding buffer to a protein concentration of 50 μg/ml. The final refolding buffer contained 3M urea/50 mM Tris-HCl (pH 8.0)/1 mM EDTA/0.3 mM oxidized glutathione/3 mM reduced glutathione/1 mM

PMSF. The refolding solution was incubated at 4°C with gentle mixing for 48 hours and then dialyzed against 100 volume of PBS at 4°C with at least 4 changes. Refolded protein was first concentrated in an Amicon stir cell with a YM10 membrane to a volume of 25-50 ml, then further concentrated by vacuum dialysis against PBS.

iii) Affinity purification of corrected folded proteins

The protein solution was cleared by passage through a 0.45 μm filter and applied to a column of Acti-gel (Sterogen) with immobilized C1 (Friedman et al., *J. Exp. Med. 174*: 891 (1991)). The column was washed with 10 volumes of PBS and the single chain T-cell receptor was eluted with 0.15 M glycine (pH 2.8). Fractions were immediately neutralized with 1/10 volume of saturated Tris. The single chain T-cell receptor was then dialyzed against more than 100 volumes of PBS at 4°C with at least 4 changes and concentrated to 0.5 mg/ml by vacuum dialysis against PBS.

iv) ELISA

For the two-antibody ELISA, βF1 (Brenner et al., *J. Immunol. 138*: 1502 (1987)) was immobilized on a 94-well ELISA plate (200 ng/well) by incubating in 50 mM bicarbonate buffer (pH 9.5) overnight at 4°C. After blocking with PBS containing 3% BSA (Sigma) and 0.05% Tween-20 at room temperature for 1 hour, single chain T-cell receptor preparations were added in serial dilution. Wells were washed with PBS containing 0.05% Tween-20, followed by the addition of biotinylated C1 antibody. Binding was detected with streptavidin-conjugated alkaline phosphatase (Sigma) after the addition of substrate (Bio-rad). Absorbence at 410 nm was determined using a 96-well plate reader. For the direct-binding ELISA, soluble T-cell receptors in serial dilution were immobilized on the plate. After the blocking step, C1 antibody was added to the wells

followed by an alkaline phosphatase conjugated anti-mouse immunoglobulin antibody. The binding was measured after the addition of the substrate as described above.

Results

The present example relates to the expression of a three-domain single chain T-cell receptor in bacteria. The final construct for bacterial expression bears a three-domain design similar to its eukaryotic counterpart described in Example 1, with a few modifications. More specifically, the N-terminal endogenous α-chain leader sequence was deleted, a histidine tag was added to the C-Terminus for convenient purification with Ni-NTA resin and additional amino acid residues created convenient restriction sites at these junction regions. This three-domain single chain T-cell receptor was cloned into a bacterial expression vector in frame with a N-terminal PelB signal sequence. The presence of the PelB sequence was intended to allow recombinant proteins to be expressed as secretory proteins and to be isolated from either the growth medium or the periplasmic space. However, it was determined that the yield from these preparations was extremely low and the majority of the proteins were accumulated in bacteria as inclusion bodies. Based on these observations, the three-domain single chain T-cell receptors were isolated from inclusion bodies. The recombinant proteins were subsequently purified with a one-step Ni-NTA chromatography procedure through the binding of histidine tag engineered at its C-terminus. SDS-PAGE analysis of the induction of the three-domain single chain T-cell receptor, the inclusion body preparation, the flowthrough of the NI-NTA column and purified proteins eluted from the column was performed. In general, 5-10 mg of the recombinant protein could be purified from each liter of bacteria culture.

The purified protein which was reduced and denatured following the NI-NTA column chromatography step was subsequently refolded with glutathione. The efficiency of refolding was evaluated with a two-antibody ELISA where the fraction of C1 reactive protein was measured. Previously, C1 had been demonstrated to recognize a $V\beta_{17}$ specific epitope. Further, it had been shown and that the formation of this epitope is dependent upon the proper folding and pairing of the T-cell receptor α and β chains. The eukaryotically expressed three-domain single chain T-cell receptor was used as a control in the two-antibody ELISA. The eukaryotically produced single chain T-cell receptor was purified from the cell surface after the PI-PLC cleavage and likely consists of T-cell receptors completely folded in their native conformation. Based on a determination of C1 reactivity between the two preparations, 5-10% of the bacterially-expressed three-domain single chain T-cell receptor was properly folded.

To confirm the importance of the Cβ domain, a two-domain single chain T-cell receptor based on the design of scFv was constructed and purified in the manner described above in connection with the three-domain single chain T-cell receptor. The folding of the two-domain T-cell receptor was assayed by direct-binding ELISAs with the C1 antibody. The bacterially expressed three-domain single chain T-cell receptor was used as a control. Refolded proteins were either directly immobilized to an ELISA plate or through the binding of Ni molecule which was coated on the bottom of the wells. The results of both studies demonstrate that, while the three-domain single chain T-cell receptor showed significant C1 reactivity, the two-domain single chain T-cell receptor was unable to interact with the C1 antibody even at high concentration. This is consistent

with observations following eukaryotic expression which highlight the importance of Cβ domain in this single chain design.

The C1 reactive fraction of the bacterially expressed three-domain single chain T-cell receptor can be further separated from the refolded protein solution using a C1-affinity column. Consistent with the result from the two-antibody ELISA, 5-10% of the protein was recovered from the C1 column. Additionally, the C1 purified three-domain single chain T-cell receptor appeared as a single band in a non-reduced SDS-PAGE, in contrast to the multiple bands observed from the refolded protein solution. Furthermore, in a two-antibody ELISA, the C1 purified protein displayed comparable C1 reactivity to the eukaryotically expressed three-domain single chain T-cell receptor preparation.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The President and Fellows of Harvard College

(ii) TITLE OF INVENTION: SINGLE CHAIN T-CELL RECEPTOR

(iii) NUMBER OF SEQUENCES: 14

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: York Harbor
(D) STATE: Maine
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(F) ZIP: 03911

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/349,915
(B) FILING DATE: 06-DEC-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Farrell, Kevin M.
(B) REGISTRATION NUMBER: 35,505
(C) REFERENCE/DOCKET NUMBER: HU-9404 WO

(ix) TELECOMMUNICATION INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1176 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1176

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu Leu Ala
 1               5                  10                  15

GCC CAA CCA GCG ATG GCG GAT CTC CAA CAG GGA GAG GAG GAT CCT CAG        96
Ala Gln Pro Ala Met Ala Asp Leu Gln Gln Gly Glu Glu Asp Pro Gln
            20                  25                  30
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GCC TTG AGC ATC CAG GAG GGT GAA AAT GCT ACC ATG AAC TGC AGT TAC        144
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          35                  40                  45

AAA ACT AGT ATA AAC AAT TTA CAG TGG TAT AGA CAA AAT TCA GGT AGA        192
Lys Thr Ser Ile Asn Asn Leu Gln Trp Tyr Arg Gln Asn Ser Gly Arg
      50                  55                  60

GGC CTT GTC CAC CTA ATT TTA ATA CGT TCA AAT GAA AGA GAG AAA CAC        240
Gly Leu Val His Leu Ile Leu Ile Arg Ser Asn Glu Arg Glu Lys His
 65                  70                  75                  80

AGT GGA AGA TTA AGA GTC ACG CTT GAC ACT TCC AAG AAA AGC AGT TCC        288
Ser Gly Arg Leu Arg Val Thr Leu Asp Thr Ser Lys Lys Ser Ser Ser
                 85                  90                  95

TTG TTG ATC ACG GCT ACC CGG GCA GCA GAC ACT GCT TCT TAC TTC TGT        336
Leu Leu Ile Thr Ala Thr Arg Ala Ala Asp Thr Ala Ser Tyr Phe Cys
            100                 105                 110

GCT ACG GAT ACA GGA GGA AGC TAC ATA CCT ACA TTT GGA AGA GGA ACC        384
Ala Thr Asp Thr Gly Gly Ser Tyr Ile Pro Thr Phe Gly Arg Gly Thr
        115                 120                 125

AGC CTT ATT GTT CAT CCG AGC TCT GGT GGC GGT GGG TCG GGC GGT GGT        432
Ser Leu Ile Val His Pro Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly
    130                 135                 140

GGC TCG GGT GGC GGC GGA TCG GAT ATC GAT GGT GGA ATC ACT CAG TCC        480
Gly Ser Gly Gly Gly Gly Ser Asp Ile Asp Gly Gly Ile Thr Gln Ser
145                 150                 155                 160

CCA AAG TAC CTG TTC AGA AAG GAA GGA CAG AAT GTG ACC CTG AGT TGT        528
Pro Lys Tyr Leu Phe Arg Lys Glu Gly Gln Asn Val Thr Leu Ser Cys
                165                 170                 175

GAA CAG AAT TTG AAC CAC GAT GCC ATG TAC TGG TAC CGA CAG GAC CCA        576
Glu Gln Asn Leu Asn His Asp Ala Met Tyr Trp Tyr Arg Gln Asp Pro
            180                 185                 190

GGG CAA GGG CTG AGA TTG ATC TAC TAC TCA GAG ATA GTA AAT GAC TTT        624
Gly Gln Gly Leu Arg Leu Ile Tyr Tyr Ser Glu Ile Val Asn Asp Phe
        195                 200                 205

CAG AAA GGA GAT ATA GCT GAA GGG TAC AGC GTC TCT CGG GAG AAG AAG        672
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    210                 215                 220

GAA TCC TTT CCT CTC ACT GTG ACA TCG GCC CAA AAG AAC CCG ACA GCT        720
Glu Ser Phe Pro Leu Thr Val Thr Ser Ala Gln Lys Asn Pro Thr Ala
225                 230                 235                 240

TTC GGG CCA GGG AAC CGG CTC ACC GTG CTA GAG GAC CTG AAA AAC GTG        768
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            260                 265                 270

CAC ACC CAA AAG GCC ACA CTG GTG TGC CTG GCC ACA GGT ATC TTC CCT        864
His Thr Gln Lys Ala Thr Leu Val Cys Leu Ala Thr Gly Ile Phe Pro
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GAC CAC GTG GAG CTG AGC TGG TGG GTG AAT GGG AAG GAG GTG CAC AGT        912
Asp His Val Glu Leu Ser Trp Trp Val Asn Gly Lys Glu Val His Ser
    290                 295                 300
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305                 310                 315                 320

GAC TCC AGA TAC TGC CTG AGC AGC CGC CTG AGG GTC TCG GCC ACC TTC       1008
Asp Ser Arg Tyr Cys Leu Ser Ser Arg Leu Arg Val Ser Ala Thr Phe
                325                 330                 335

TGG CAG AAC CCC CGC AAC CAC TTC CGC TGT CAA GTC CAG TTC TAC GGG       1056
Trp Gln Asn Pro Arg Asn His Phe Arg Cys Gln Val Gln Phe Tyr Gly
            340                 345                 350

CTC TCG GAG AAT GAC GAG TGG ACC CAG GAT AGG GCC AAA CCC GTC ACC       1104
Leu Ser Glu Asn Asp Glu Trp Thr Gln Asp Arg Ala Lys Pro Val Thr
        355                 360                 365

CAG ATC GTC AGC GCC GAG GCC TGG GGT AGA GCA GAT ATC GAG GGT AGG       1152
Gln Ile Val Ser Ala Glu Ala Trp Gly Arg Ala Asp Ile Glu Gly Arg
    370                 375                 380

ATC CAT CAC CAT CAC CAT CAC TAA                                      1176
Ile His His His His His His
385                 390
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 391 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu Leu Ala
  1               5                  10                  15

Ala Gln Pro Ala Met Ala Asp Leu Gln Gln Gly Glu Glu Asp Pro Gln
            20                  25                  30

Ala Leu Ser Ile Gln Glu Gly Glu Asn Ala Thr Met Asn Cys Ser Tyr
        35                  40                  45

Lys Thr Ser Ile Asn Asn Leu Gln Trp Tyr Arg Gln Asn Ser Gly Arg
    50                  55                  60

Gly Leu Val His Leu Ile Leu Ile Arg Ser Asn Glu Arg Glu Lys His
65                  70                  75                  80

Ser Gly Arg Leu Arg Val Thr Leu Asp Thr Ser Lys Lys Ser Ser Ser
                85                  90                  95

Leu Leu Ile Thr Ala Thr Arg Ala Ala Asp Thr Ala Ser Tyr Phe Cys
            100                 105                 110

Ala Thr Asp Thr Gly Gly Ser Tyr Ile Pro Thr Phe Gly Arg Gly Thr
        115                 120                 125

Ser Leu Ile Val His Pro Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly
    130                 135                 140

Gly Ser Gly Gly Gly Gly Ser Asp Ile Asp Gly Gly Ile Thr Gln Ser
145                 150                 155                 160
```

```
Pro Lys Tyr Leu Phe Arg Lys Glu Gly Gln Asn Val Thr Leu Ser Cys
                165                 170                 175

Glu Gln Asn Leu Asn His Asp Ala Met Tyr Trp Tyr Arg Gln Asp Pro
            180                 185                 190

Gly Gln Gly Leu Arg Leu Ile Tyr Tyr Ser Glu Ile Val Asn Asp Phe
        195                 200                 205

Gln Lys Gly Asp Ile Ala Glu Gly Tyr Ser Val Ser Arg Glu Lys Lys
    210                 215                 220

Glu Ser Phe Pro Leu Thr Val Thr Ser Ala Gln Lys Asn Pro Thr Ala
225                 230                 235                 240

Phe Gly Pro Gly Asn Arg Leu Thr Val Leu Glu Asp Leu Lys Asn Val
                245                 250                 255

Phe Pro Pro Glu Val Ala Val Phe Glu Pro Ser Glu Ala Glu Ile Ser
            260                 265                 270

His Thr Gln Lys Ala Thr Leu Val Cys Leu Ala Thr Gly Ile Phe Pro
        275                 280                 285

Asp His Val Glu Leu Ser Trp Trp Val Asn Gly Lys Glu Val His Ser
    290                 295                 300

Gly Val Ser Thr Asp Pro Gln Pro His Lys Glu Gln Pro Ala Leu Asn
305                 310                 315                 320

Asp Ser Arg Tyr Cys Leu Ser Ser Arg Leu Arg Val Ser Ala Thr Phe
                325                 330                 335

Trp Gln Asn Pro Arg Asn His Phe Arg Cys Gln Val Gln Phe Tyr Gly
            340                 345                 350

Leu Ser Glu Asn Asp Glu Trp Thr Gln Asp Arg Ala Lys Pro Val Thr
        355                 360                 365

Gln Ile Val Ser Ala Glu Ala Trp Gly Arg Ala Asp Ile Glu Gly Arg
    370                 375                 380

Ile His His His His His His
385                 390
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Leu Ala Pro Pro Ala Gly Thr Thr Asp Ala Ala His Pro Gly Arg Ser
1               5                   10                  15

Val Val Pro Ala Leu Leu Pro Leu Leu Ala Gly Thr Leu Leu Leu Leu
            20                  25                  30
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCTCGAGGCG GCGATGGAAA CTCTCCTGGG AGT 33

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGAATTCAGC TGGACCACAG CCGC 24

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCTCGAGCTC TGCCATGGAC TCCTGGA 27

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGAATTCAGA AATCCTTTCT CTTGAC 26

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CAGAGCTCAC GGATGAACAA TAAGGCTGGT 30

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCGGATATCG ATGGTGGAAT CACTCAGTCC 30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CAGAGATCAG CACGGTGAGC CGGTTCCC 28

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTGGGAGATC TCTGCTTCTG ATGGCTCAAA C 31

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CACGGATCCC CGTCTGCTCT ACCCCAGGC 29

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CACGGATCCC CGTCTGCTCT ACCCCAGGC 29

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
1 5 10 15

CLAIMS

1. A single chain T-cell receptor which binds specifically to an MHC/peptide ligand.

2. The single chain T-cell receptor of Claim 1 wherein the MHC/peptide ligand is located on the surface of an antigen presenting cell.

3. The single chain T-cell receptor of Claim 1 which is soluble in an aqueous solution.

4. The single chain T-cell receptor of Claim 1 which is a 3-domain single chain T-cell receptor comprising an α chain variable domain, a β chain variable domain and a constant domain.

5. The single chain T-cell receptor of Claim 4 wherein the Vα-Jα domain of the α chain variable domain is linked to the N-terminus of the β chain variable domain by a flexible amino acid linker and the C-terminal region of the β chain variable domain is linked to the β chain constant domain.

6. A self-signaling single chain T-cell receptor which binds specifically to an MHC/peptide ligand, the self-signaling T-cell receptor comprising:
   a) a soluble T-cell receptor domain comprising an α chain variable domain, a β chain variable domain and a constant domain; and
   b) a transmembrane and intracellular signaling domain from a transmembrane receptor.

7. The self-signaling single chain T-cell receptor of Claim 6 wherein the Vα-Jα domain of the α chain variable domain is linked to the N-terminus of the β chain variable domain by a flexible amino acid linker; the C-terminal region of the β chain variable domain is linked to the β chain constant domain; and the C-terminal region of the β chain constant region is linked to the N-terminal portion of the transmembrane and intracellular signaling domain of the transmembrane receptor.

8. The self-signaling single chain T-cell receptor of Claim 6 wherein the transmembrane receptor is selected from the group consisting of CD3 ζ-chain or Fc_γ.

9. A nucleic acid molecule encoding a single chain T-cell receptor which binds specifically to an MHC/peptide ligand.

10. A nucleic acid molecule of Claim 9 which is soluble in an aqueous solution.

11. A nucleic acid molecule of Claim 9 which encodes a 3-domain single chain T-cell receptor comprising an α chain variable domain, a β chain variable domain and a constant domain.

12. The nucleic acid molecule of Claim 9 wherein the single chain T-cell receptor comprises the Vα-Jα domain of the α chain variable domain is linked to the N-terminus of the β chain variable domain by a flexible amino acid linker and the C-terminal region of the β chain variable domain is linked to the β chain constant domain.

13. A DNA expression construct comprising, in expressible form, a nucleic acid molecule encoding a single chain T-cell receptor which binds specifically to an MHC/peptide ligand.

14. The DNA expression construct of Claim 13 wherein the single chain T-cell receptor comprises the Vα-Jα domain of the α chain variable domain linked to the N-terminus of the β chain variable domain by a flexible amino acid linker and the C-terminal region of the β chain variable domain linked to the β chain constant domain.

15. A eukaryotic cell transformed with a DNA expression construct, the DNA expression construct comprising, in expressible form, a nucleic acid molecule encoding a single chain T-cell receptor which binds specifically to an MHC/peptide ligand.

16. A prokaryotic cell transformed with a DNA expression construct, the DNA expression construct comprising, in expressible form, a nucleic acid molecule encoding a single chain T-cell receptor which binds specifically to an MHC/peptide ligand.

17. A nucleic acid molecule encoding a self-signaling single chain T-cell receptor which binds specifically to an MHC/peptide ligand, the self-signaling single chain T-cell receptor comprising an α chain variable domain, a β chain variable domain, a constant domain and a transmembrane and intracellular signaling domain from a transmembrane receptor.

18. A DNA expression construct comprising, in expressible form, a nucleic acid molecule encoding a self-signaling single chain T-cell receptor which binds specifically to an MHC/peptide ligand, the self-signaling single chain T-cell receptor comprising:
    a) a soluble T-cell receptor domain comprising an α chain variable domain, a β chain variable domain and a constant domain; and
    b) a transmembrane and intracellular signaling domain from a transmembrane receptor.

19. A eukaryotic cell transformed with a DNA expression construct comprising, in expressible form, a nucleic acid molecule encoding a self-signaling single chain T-cell receptor which binds specifically to an MHC/peptide ligand, the self-signaling single chain T-cell receptor comprising:
    a) a soluble T-cell receptor domain comprising an α chain variable domain, a β chain variable domain and a constant domain; and
    b) a transmembrane and intracellular signaling domain from a transmembrane receptor.

20. The eukaryotic cell of Claim 19 which is a T-cell.

21. The eukaryotic cell of Claim 19 which is a killer T-cell.

22. A method for diagnosing viral infection in a patient, comprising:
    a) providing a soluble T-cell receptor which binds specifically to an MHC/peptide ligand wherein the peptide component is a viral specific T-cell epitope;

b) incubating the soluble T-cell receptor of step a) with antigen presenting cells isolated from the patient under conditions appropriate for binding of the soluble T-cell receptor to the MHC/peptide ligand; and

c) detecting specific binding of the soluble T-cell receptor to the MHC/peptide complex on the surface of the antigen presenting cells, specific binding being indicative of viral infection.

23. A method for diagnosing infection using a cell bearing chimeric transmembrane receptor comprising a 3-part single chain T-cell receptor fused to a transmembrane region and an intracellular signaling domain from a transmembrane receptor, comprising:

a) providing antigen presenting cells from a patient;

b) fixing the antigen present-cells with a fixing agent;

c) providing a self-signaling T-cell clone which binds to a predetermined antigen, the self signaling T-cell clone comprising a T-cell bearing a self-signaling T-cell receptor on its cell surface, the self-signaling T-cell receptor comprising a 3-part T-cell receptor fused to a transmembrane region and an intracellular signaling domain from a transmembrane receptor;

d) incubating the fixed antigen presenting cells from step b) and the self-signaling T-cell clone of step c) under conditions appropriate for T-cell receptor binding to MHC/peptide ligand; and

e) detecting signal as an indication of the presence of the predetermined antigen on the surface of the antigen presenting cells in association with MHC.

24. The method of Claim 23 wherein the T-cell clone is derived from a normal T-cell isolated from the patient.

25. The method of Claim 23 wherein the T-cell clone is derived from a benign tumor cell line.

26. The method of Claim 23 wherein the MHC is type I or type II.

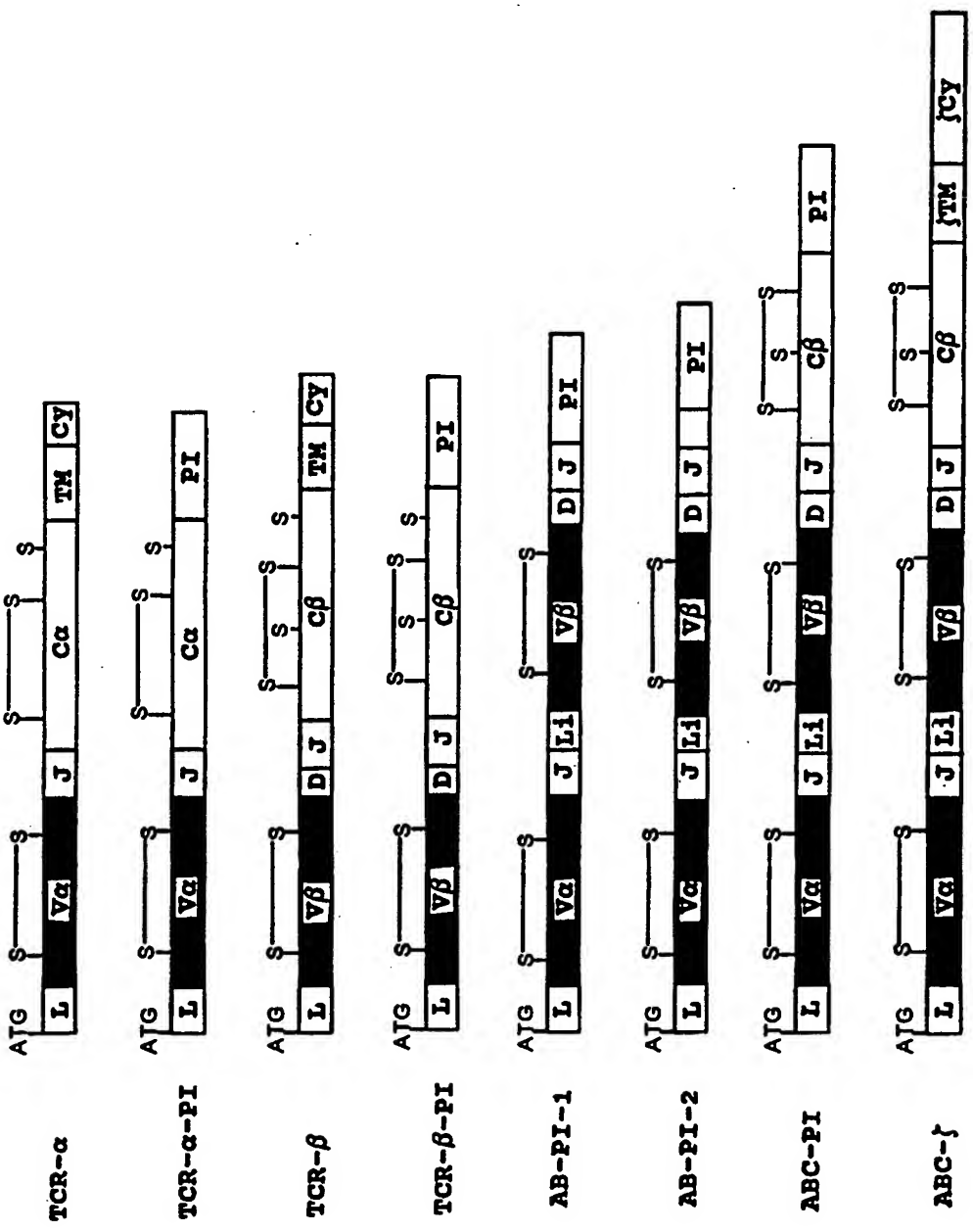
TCR-α
TCR-α-PI
TCR-β
TCR-β-PI
AB-PI-1
AB-PI-2
ABC-PI
ABC-ϳ
ATG
L
Vα
Cα
J
TM
Cy
PI
Vβ
Cβ
D
Li
ϳTM
ϳCy
S

FIGURE 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/15696

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :435/7.2, 69.7, 240.2, 252.3, 320.1; 536/23.4; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.2, 69.7, 240.2, 252.3, 320.1; 536/23.4; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, World Patents Index
search terms: T cell receptors, TCR, fusion, chimer?, single chain

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Proceedings of the National Academy of Science USA, Volumn 91, September 1994, V HILYARD et al., "Binding of Soluble Natural Ligands to a Soluble Human T-cell Receptor Fragment Produced in Escherichia coli", pages 9057-9061, see especially pages 9058-9060.	1-3, 9, 10, 13, 16 --- 6-8, 17-21
X --- Y	WO, A, 91/18019 (E.R. SQUIBB AND SONS, INC.) 28 November 1991, pages 6, 10, 13, 19 and 40-44.	1-3, 9, 10, 13, 15, 16, 22 --- 6-8, 17-21, 23-26

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 MARCH 1996	28 MAR 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KAREN E. BROWN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/15696

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,359,046 (CAPON et al.) 25 October 1994, column 8, line 11 to column 12, line 25.	6-8, 17-21, 23-26
Y	Annual Review of Biochemistry, Volumn 59, 1990, DAVIS, "T CELL RECEPTOR GENE DIVERSITY AND SELECTION" pages 475-496, see especially pages 478-479 and Figures 2 and 3.	6-8, 17-21, 23-26
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A		4, 5, 11, 12, 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/15696

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

G01N 33/566; C12P 21/02; C12N 1/21, 5/10, 15/12, 15/63, 15/70, 15/85; C07K 14/725

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